Galleria mellonella as an infection model for *Campylobacter jejuni* virulence

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Running title: *C. jejuni* insect model

Models of infection

Summary

Larvae of *Galleria mellonella* (Greater wax moth) have been shown to be susceptible to *Campylobacter jejuni* infection and our study characterises this infection model. Following infection with *C. jejuni* human isolates, bacteria were visible in the hemocoel and gut of challenged larvae, and there was extensive damage to the gut. Bacteria were found in the extracellular and cell associated fraction in the hemocoel, and we show that *C. jejuni* can survive in insect cells. Finally, we have used the model to screen a further sixty-seven *C. jejuni* isolates belonging to different MLST types. Isolates belonging to ST257 were the most virulent, whereas those belonging to ST21 were the least virulent in the *Galleria* model.

Introduction

Campylobacter jejuni is recognised as the leading cause of bacterial gastroenteritis across the developed world; the World Health Organisation estimates that 1% of the population of Western Europe is infected with campylobacters each year. It is thought that for each reported case, a further nine go unreported (Humphrey *et al.*, 2007), thus, based on the reported figures for 2009 from the Health Protection Agency, this would mean there were in excess of 500,000 cases in England and Wales alone. Furthermore, beyond the initial diarrhoeal disease, *C. jejuni* may also cause post-infection complications including irritable bowel syndrome, meningitis and Guillain-Barré Syndrome, plus its variant Miller-Fisher Syndrome (Janssen *et al.*, 2008; van Doorn *et al.*, 2008).

Despite having been first identified as a causative agent of diarrhoea in 1977 (Skirrow, 1977), *C. jejuni* pathobiology remains poorly understood, with its core virulence determinants remaining elusive. A major contributing factor in the lack of determination of these factors has been the absence of a suitable infection model for *C. jejuni*. Previous models have included a ferret diarrhoeal model (Fox *et al.*, 1987), a chick colonisation model (Wassenaar *et al.*, 1993) and a colostrum deprived piglet model (Babakhani *et al.*, 1993). However, in common with other mammalian and avian models, their widespread use has been limited by factors such as cost, ease of use, reproducibility and ethics (Newell, 2001).

We recently reported that larvae of the lepidopteran insect *Galleria mellonella* (Greater wax moth) are susceptible to infection by *C. jejuni* and can be used to screen for virulence genes (Champion *et al.*, 2010). In this study we aimed to characterise *G. mellonella* as a *C. jejuni* infection model and to screen a panel of multi locus sequence typed (MLST) *C. jejuni* field isolates for virulence in *G. mellonella*. Different MLST groups were chosen to cover types detected in the main food producing animals, the environment and clinical disease.

Methods

Strains and cultures

All bacterial strains and mutants used in this study are shown in Table 1. *C. jejuni* strain 11168H is a hypermotile variant of the sequenced strain NCTC11168 that readily colonises chickens *(*Jones *et al.*, 2004; Karlyshev *et*

al., 2002). *C. jejuni* strains were cultured on either blood agar Skirrows actidione (BASA) plates or Columbia agar plates (CBA) supplemented with 5% (v/v) horse blood in anaerobic jars in an atmosphere of 6% O_2/10% CO_2 conditions (CampyPak, Oxoid) for 48 hours at 37ºC.

For infections, bacteria were subcultured into 6ml of Mueller-Hinton (MH) broth (Oxoid) and grown under microaerobic conditions for 24 to 48 hours at 37ºC, 150rpm. The bacteria were then adjusted to OD_{590nm} 1.0 in phosphate-buffered saline (PBS, 0.1M, pH 7.2) for infections, equivalent to 1 x 10^8 cfu/ml. Infections at lower doses were adjusted accordingly.

G. mellonella virulence assays

G. mellonella larvae were purchased from Live Foods UK and maintained on wood chips at 15°C. The infection of larvae was carried out as previously described (Champion *et al.*, 2010) using a micro-injection technique whereby 10µl of *C. jejuni* was injected into the hemocoel via the right fore leg, using a Hamilton syringe. Larvae were then incubated at 37^{o}C and survival and macroscopic appearance recorded at 24 hours post-infection. PBS injected and uninfected controls were used. For each, experimental groups of ten *G. mellonella* larvae were infected.

The association of _C. jejuni_ with hemocytes

A group of three *G. mellonella* larvae was infected as above with 10^6 cfu of *C. jejuni* 11168-H and incubated at 37ºC for 24 hours. The larvae were chilled on ice for 20 minutes before aseptic removal of the bottom 2mm of the body.

The hemocoel was drained from each larva into sterile microcentrifuge tubes and centrifuged at 200 x *g* for 5 minutes. The supernatant, which was the hemolymph, was transferred to a separate sterile microcentrifuge tube. The pelleted hemocytes were resuspended in 100µl of sterile distilled water, and pipetted up and down ten times to lyse the cells. Serial dilutions of both hemolymph and hemocytes were plated out on CBA to enumerate bacteria.

Histopathology

C. jejuni-infected and uninfected larvae (5 per group) were fixed by immersion in 10% (v/v) neutral buffered formalin for 3-7 days. For light microscopy, larvae were blocked by a longitudinal section dividing the animal into two pieces and smears were air-dried and stained with Gram-Twort. The larvae (20%) were blocked into eleven transversal sections serially from the cranial to the caudal extremities of the larvae. Sections were embedded in paraffin wax and routinely stained with Haematoxylin and Eosin (H&E) for microscopic examination.

Investigation of *C. jejuni* morphology following infection of *G. mellonella*

A GFP-tagged *C. jejuni* strain, pREM5 11168H GFP (donated by Andrey Karlyshev) was cultured under microaerobic conditions on MH agar. It was then subcultured into MH broth as before and incubated for 24 hours at 37ºC under microaerobic conditions. An inoculum was prepared at OD_{590nm} as previously described above. Five *G. mellonella* larvae were infected with 10 µl of the prepared inoculum, and a further five were inoculated with 10 µl of PBS. The larvae were incubated at 37ºC for 3 hours before being chilled on

ice for 5 minutes. They were then swabbed with 70% ethanol prior to the aseptic removal of the bottom 2mm of the body as previously described. One of each larval set was drained separately; the other 4 of each set had their hemocoel combined. This combined hemocoel was centrifuged at 500 x *g* for 5 minutes to pellet the hemocytes, and 10 µl of the supernatant (hemolymph) was dropped onto a slide. A further 10 µl from the non-centrifuged hemocoel and 10 µl from the overnight *C. jejuni* culture were also dropped onto separate slides. Slides were examined using a Zeiss LSM 510 META confocal microscope.

Cell culture

J774A.1, a murine monocyte macrophage-like cell line, was obtained from the American Type Culture Collection (ATCC), (Reference TIB-67), and was cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37ºC. SF9, a lepidopteran cell line, was donated by Richard ffrench-Constant, and cultured in Grace's Insect Medium (GIM) supplemented with 10% fetal bovine serum at 27ºC. The cells were seeded at 2 x 10^5 cells in 6-well tissue culture plates and then incubated at the appropriate temperature for 24 hours under 5% CO_2 prior to infection with *C. jejuni*.

Bacterial infection of cultured cells

C. jejuni 11168-H was cultured on a CBA plate and harvested from an overnight culture by rolling a moistened swab over the plate; cells were re-suspended in PBS. OD_{590nm} was measured and the inoculum was prepared

at a Multiplicity of Infection (MOI) of 10 in L-15 medium before being added to both macrophages and insect cells (3 replicates). The macrophages were incubated at 37ºC for 1 hour; the insect cells were incubated at 27ºC for 1 hour. Following incubation, the inoculum in each well was replaced with L-15 medium containing 50 µg ml^{-1} gentamicin, and the plates then incubated at the appropriate temperature for a further 1 hour. The medium was removed and the cells incubated in L-15 containing 10 µg ml^{-1} gentamicin for approximately 16 hours.

The cells were then washed x3 with PBS, and 1ml cold sterile water was added to each well. The cells were mechanically lysed to release intracellular bacteria, and colony forming units (cfu) were determined after plating out serial dilutions on CBA plates and incubating microaerobically at 37ºC.

Results

C. jejuni induces histopathological changes in *G. mellonella*

In order to better understand the fate of *C. jejuni* inoculated into *G. mellonella*, larvae challenged with 10^6 cfu of some well-characterised human isolates were fixed in neutral buffered formalin at 24 hours post-infection and sectioned for histopathology. Figure 1 shows H and E stained sections of uninfected and infected larvae. Bacteria were observed in the hemocoel and sections of gut from infected larvae, but were absent in sections from uninfected controls. There was evidence of damage to the midgut, with apoptotic cells and loss of integrity to the gut wall in the infected larvae. This damage was not visible in the control sections. Other tissues (fat body,

muscle, nervous tissue) appeared undamaged in infected larvae and uninfected controls. Pigmented nodules were also present in infected larvae, and bacteria were associated with these nodules. These nodules were not visible in the uninfected control larvae.

The observed bacteria were coccoid rather than having the characteristic spiral form associated with *C. jejuni*. In order to investigate whether these coccoid bacteria were actually *C. jejuni*, *G. mellonella* larvae were infected with GFP-tagged *C. jejuni*; the hemocoel was collected and centrifuged at low speed to sediment hemocytes, which are often auto-fluorescent. Comparisons with *C. jejuni* from an overnight culture showed that these bacteria had the expected morphology, but bacteria in hemolymph were of a coccoid nature (Figure 2). Similar observations of hemolymph from control larvae inoculated with PBS showed no fluorescence at all.

To investigate the site of replication, hemocoel was collected and centrifuged at low speed to sediment hemocytes. The number of bacteria found in the re-suspended cell pellet (4.1×10^6 cfus; s.e.m 2.98×10^6) was broadly similar to the number found in the hemolymph (7.7×10^6 cfus; s.e.m 2.3×10^6).

A macro scoring system was used to examine whether there was a correlation between the colour of the larvae and the presence of bacteria in the gut or body cavity (Table 2; Figure 3). There was a significant association between macro colour and the presence of bacteria in the body cavity ($p < 0.001$, Kruskall-Wallis non-parametric test). No associations were made between the

location of the bacteria and temperature at which it had been grown, or location and the strain of *C. jejuni* used to inoculate the larvae.

An insect cell line and mammalian macrophages are comparable in their response to challenge with *C. jejuni*

Insect (SF9) and mammalian (J774A.1) cells were infected at an MOI of 10 with *C. jejuni* 11168-H, and monitored at 4 hours and 24 hours post-infection. In J774A.1 macrophages, bacterial numbers declined 100-fold by 4 hours post-infection (Figure 4). However, the bacterial numbers then remained approximately constant in the macrophages at 24 hours post-infection. There was a broadly similar pattern of survival in the SF9 cell line. Bacterial numbers decreased 1000-fold during the first 4 hours of the infection, but there was an approximate 10-fold increase in bacterial numbers between 4 hours and 24 hours (Figure 4). This increase was statistically analysed using a Student's t-test and found to be significant ($p < 0.05$).

Differences in virulence were observed in *G. mellonella* between *C. jejuni* Multi-Locus Sequence Typing (MLST) complexes

To investigate whether there was an association between MLST type and virulence, larval survival was recorded following challenge with sixty-seven *C. jejuni* strains belonging to different MLST types (Figure 5). It was observed that there was variation within MLST groups as well as between them. There was a significant difference ($p = 0.0002$) between the ability of ST21 and ST257 strains to cause disease. Overall, strains belonging to ST21 showed

248 the least virulence in the model, whilst strains belonging to ST257 were the
249 most virulent.
250
251 **Discussion**
252 We have previously demonstrated that *G. mellonella* larvae can be used to
253 screen for virulence of *Campylobacter* genes (Champion *et al.*, 2010). In this
254 study we have characterised the *G. mellonella* model and demonstrated that it
255 can be used as an infection model to provide data about pathology and
256 intracellular survival.
257
258 Similarities between invertebrate and mammalian humoral and cellular innate
259 immune responses are exploited when using insects as virulence models. For
260 example, *G. mellonella* possesses a cuticle that acts in the same physical
261 barrier capacity as mammalian skin (Kemp & Massey, 2007). Once the
262 cuticle has been breached, *G. mellonella* induces a humoral response,
263 producing soluble factors such as antimicrobial peptides (Mullett *et al.*, 1993).
264 In parallel with a humoral response, *G. mellonella* induces a cellular response
265 to invading micro-organisms. Insect hemocytes phagocytose bacteria in a
266 manner similar to that of mammalian neutrophils and produce a respiratory
267 burst (Bergin *et al.*, 2005). Thus, the response of *G. mellonella* to infection
268 with *C. jejuni* is likely to have similarities to the response of humans.
269
270 In the initial experiment, larvae were infected with human *C. jejuni* isolates
271 11168-H, 11168-O, 81116, 81176 and 01/51. These were selected as they
272 are well-characterised in a number of other animal models; invasion and toxin

data are also available for them. It would not have been possible to fix *G. mellonella* for all the strains used later on, as this would have been time consuming and expensive with no guarantee of any further data.

Histopathology of infected larvae demonstrated that bacteria are found in the hemocoel and in the gut and that extensive tissue damage occurs in the latter. This pathology may be caused by hemocytes in the gut tissue, which have ingested bacteria circulating in the hemocoel and then produced responses such as the release of free radicals and peroxide, causing the visible tissue damage. The presence of pigmented nodules, which are aggregations of hemocytes around foreign bodies, indicates a vigorous immune response to infection (Lackie, 1980). The observed colour change in infected larvae correlating with the presence of bacteria in the body cavity is a product of melanogenesis; this process is thought to protect endogenous tissues within the cavity from systemic damage resulting from pathogen killing (Nappi & Christensen, 2005).

The bacteria observed in the larval sections were not identified, but were only present in *Campylobacter* infected larvae. The bacterial cells were coccoid rather than spiral; however, *C. jejuni* that have become intracellular convert rapidly from the spiral form to the coccoid form (Kiehlbauch *et al.*, 1985). There is some debate about how this change affects the bacteria (Moore, 2001). Adaptation to the coccoid form is generally seen as a response to stress, such as starvation or oxidative stress (Harvey & Leach, 1998). Some studies, such as Moran & Upton (1986), have reported that the coccoid form is

thus degenerative. However, it has also been reported that coccoid *C. jejuni* become viable but non-culturable, with the potential to still act as an infectious agent (He & Chen, 2010). To ascertain whether these coccoid cells were likely to be *C. jejuni*, a comparison was made between GFP-tagged *C. jejuni* grown in broth overnight versus the same bacteria inoculated into *G. mellonella* and incubated for 3 hours. Under confocal microscopy, the fluorescing bacteria from broth were seen to be elongated, reflecting the normal spiral morphology of *C. jejuni*; the bacteria from the larval hemolymph were short and round like the coccoid bacteria seen in the larval sections. Hemolymph from PBS control larvae contained no fluorescing bacteria. This observation suggests that the coccoid bacteria are indeed *C. jejuni*.

Within the hemocoel, cell-associated and free bacteria were found. *C. jejuni* is primarily an extracellular pathogen; however, intracellular survival has been hypothesised to play an important role in its pathogenesis (Kiehlbauch *et al.*, 1985; Hickey *et al.*, 2005; Young *et al.*, 2007). Reproducible *in vitro* infection models that mimic pathogenesis *in vivo* have been used to study *C. jejuni* intracellular survival in epithelial cells (De Melo *et al.*, 1989; Watson & Galan, 2008). However, reports of *C. jejuni* intramacrophage survival *in vitro* are conflicting. Some groups indicate that *C. jejuni* is killed by macrophages (Watson & Galan, 2008); others suggest that the bacteria survive within the macrophage (Day *et al.*, 2000; Hickey *et al.*, 2005). In this study, bacterial infection of different cell lines was undertaken to establish whether there was a difference in response between mammalian macrophages and an insect cell line. It was uncertain as to whether *C. jejuni*

would survive intracellularly in the SF9 insect cell line under tissue culture conditions. However, although the levels of *C. jejuni* recovered from the insect cell line were approximately ten times lower than those recovered from murine macrophages at 4 hours post-infection, it is clear from the data presented here that the bacteria did invade the cells and survive within them.

The bacterial numbers recovered from the macrophages remained consistent between 4 hours and 24 hours; there was survival within the macrophages. This is consistent with previous studies (Kiehlbauch *et al.*, 1985; Hickey *et al.*, 2005). However, there was a significant increase in recovered *C. jejuni* from the insect cell line at 24 hours compared to 4 hours. Thus, it is possible that the bacteria not only survived within the cells, but also replicated.

These observations are consistent with the hypothesis that *C. jejuni* enters insect hemocytes during *in vivo* model infections of *G. mellonella*. This intracellular persistence may allow the bacteria to avoid, or at least reduce the impact of, host antimicrobial defences. Nevertheless, the fact that at least some bacteria provoked the formation of melanised nodules is not surprising as it has previously been shown that nodule formation is associated with phagocytosis (Dean *et al.*, 2004).

C. jejuni strains can be classified by MLST complexes. A number of studies have sought to establish whether there is a link between MLST type and the development of post-infectious complications (Dingle *et al.*, 2001; Nielsen *et al.*, 2009; Islam *et al.*, 2009). It was noted that the ST-22 complex is

overrepresented in isolates from patients who have contracted Guillain-Barré Syndrome; no Guillain-Barré related isolates have been shown to carry ST-45, despite it being a common sequence type (Dingle *et al.*, 2001; Nielsen *et al.*, 2009). No sequence types have been found to be exclusive for clinical outcomes (Islam *et al.*, 2009). This supports the findings of Manning *et al.* (2003), who studied a large number of *C. jejuni* isolates and found that in terms of MLST types, the populations of veterinary and human isolates overlapped; it was suggested that most veterinary sources should be considered reservoirs of pathogenic campylobacters. However, these studies did not assess whether bacteria from different MLST types exhibited different levels of virulence. Recent studies have suggested that there may be associations between *C. jejuni* MLST type and virulence factors (Habib *et al.*, 2009; de Haan *et al.*, 2010). We observed that when bacteria selected as representatives of major MLST groups were put through the *G. mellonella* model, MLST type 257 strains were significantly more virulent than the MLST type 21 set. MLST type 257 is mainly associated with poultry and clinical isolates. MLST type 21 is common in all food producing animals; the strains used here are also all of clonal complex (CC) 21, which is one of the four most common CCs in human disease. The reduced virulence of these isolates in the model may thus appear anomalous, but Habib *et al.* (2010) suggest that the abundant prevalence of *C. jejuni* of CC-21 may be a result of its increased tolerance of stresses encountered during the human food chain. A less virulent but more stress tolerant strain would thus be encountered more frequently than a more virulent strain that did not tolerate such stresses to the same extent. The convenience of the *G. mellonella* model allows for high

throughput screening to assay for the differences in virulence. Such a model could provide preliminary data when considering food security issues.

This study has sought to further characterise *G. mellonella* as a model for *C. jejuni* infection, and suggests that, since the bacteria convert to a coccoid form once within the insect, it may be used to provide opportunities for further study of this morphological change. The model may also prove useful in investigating the *in vivo* intracellular survival of *C. jejuni* within macrophages, an area of some dispute. In particular, the model allows screening for natural variations in the virulence of *C. jejuni* field isolates, which would prove invaluable for tracking particularly virulent strains in the food chain.

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Tables

Strain	Origin	MLST group
11168-H	Human	ST43/CC21
11168-O	Human	ST43/CC21
pREM5 11168H	Human	ST43/CC21
81116	Human	ST267/CC283
81176	Human	ST913/CC42

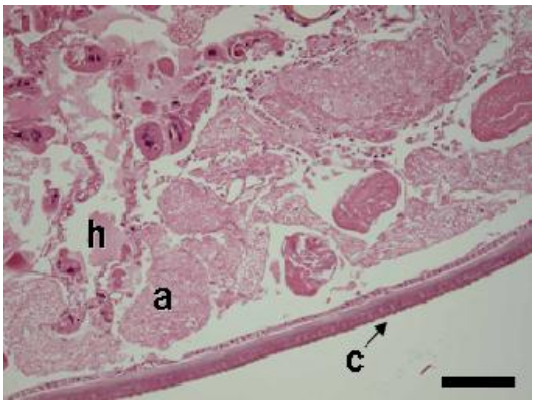
01/51	Human	ST626/CC21
01/43	Human	ST257/CC257
93/372	Pet	ST21/CC21
94/229	Poultry	ST45/CC45
99/97	Human	ST45/CC45
99/118	Cow	ST21/CC21
99/188	Human	ST21/CC21
99/189	Human	ST45/CC45
99/194	Cow	ST45/CC45
99/197	Human	ST45/CC45
99/201	Cow	ST48/CC48
99/202	Cow	ST45/CC45
99/212	Human	ST45/CC45
99/216	Human	ST45/CC45
99/242	Poultry	ST45/CC45
A1/CF/12	Poultry	ST257/CC257
A6/T2/15	Poultry	ST257/CC257
A8/35/15A	Poultry	ST257/CC257
C1/C/2	Poultry	ST257/CC257
C120/2	Poultry	ST257/CC257
C132/1	Poultry	ST19/CC21
C3/T/25	Poultry	ST257/CC257
C5/T2/8	Poultry	ST257/CC257
C85-4-99-5	Cow	ST262/CC21
C500-1-99-2	Cow	ST48/CC48
C559-3-99-2	Cow	ST262/CC21
D2/27/3	Poultry	ST48/CC48
D2/T/8	Poultry	ST48/CC48
D2/T/95	Poultry	ST48/CC48
D5-20-9A	Poultry	ST262/CC21
EX1182	Environmental	ST262/CC21
EX1286	Poultry	ST262/CC21
MB1	Poultry	ST48/CC48
MB2	Poultry	ST48/CC48
MB3	Poultry `	ST48/CC48
MB4	Poultry	ST19/CC21
MB5	Poultry	ST19/CC21
MB6	Poultry	ST19/CC21
MB7	Poultry	ST262/CC21
MB8	Poultry	ST48/CC48
MB9	Poultry	ST257/CC257
MB10	Poultry	ST19/CC21
MB12	Poultry	ST21/CC21
MB13	Poultry	ST21/CC21
MB14	Poultry	ST21/CC21
MB15	Poultry	ST45/CC45
MB16	Poultry	ST48/CC48
MB17	Poultry	ST262/CC21
MB18	Poultry	ST21/CC21
Ps308	Pig	ST51/CC403
Ps549.1	Pig	ST403/CC403
Ps623	Pig	ST552/CC403
Ps762	Pig	ST270/CC403
Ps830	Pig	ST403/CC403
Ps838	Pig	ST403/CC403
Ps843	Pig	ST403/CC403
Ps849	Pig	ST403/CC403
Ps852	Pig	ST270/CC403
Ps857	Pig	ST270/CC403
S39-2-99-3	Sheep	ST21/CC21
S87-4-99-3	Sheep	ST262/CC21
S120-4-99-4	Sheep	ST45/CC45
S216-5-99-1	Sheep	ST257/CC257
S372-5-99-4	Sheep	ST21/CC21
S379-8-99-1	Sheep	ST262/CC21
S435-3-99	Sheep	ST262/CC21
S499-1-99-5	Sheep	ST19/CC21
S585-3-99	Sheep	ST19/CC21

Table 1: A table describing the bacterial strains used in this study

C. jejuni strain	Incubation temperature	Animal number	Macro colour	Bacteria in gut	Bacteria in cavity	Mean *Galleria* survival
01/51	37ºC	1	0	3	0	80%
		2	2	3	3	
		3	3	3	3	
		4	3	3	3	
		5	3	2	2	
	42ºC	1	2	3	3	82%
		2	2	3	2	
		3	2	2	1	
		4	2	3	3	
		5	2	3	3	
11168-O	37ºC	1	1	2	3	27%
		2	1	3	3	
		3	2	3	3	
		4	2	3	3	
		5	3	3	3	
	42ºC	1	1	3	2	80%
		2	2	2	2	
		3	2	1	2	
		4	2	3	3	
		5	2	3	3	
11168-H	37ºC	1	1	2	3	0%
		2	1	2	1	
		3	2	3	3	
		4	2	1	0	
		5	3	3	2	
	42ºC	1	0	2	0	ND
		2	1	3	1	
		3	1	2	1	
		4	2	3	1	
		5	3	3	3	
81116	37ºC	1	1	2	0	90%
		2	1	3	0	
		3	3	3	3	
		4	3	3	3	
		5	3	3	3	
	42ºC	1	1	2	2	65%
		2	1	2	0	
		3	2	3	2	
		4	2	2	0	
		5	3	1	3	
81176	37ºC	1	1	2	0	90%
		2	1	2	0	
		3	2	2	0	
		4	2	2	1	
		5	3	2	3	
	42ºC	1	2	2	2	75%
		2	2	3	3	
		3	2	3	3	
		4	3	2	3	
		5	3	3	3	
PBS		1	0	1	0	100%
		2	0	2	0	
		3	0	2	0	
		4	0	2	0	
		5	1	1	0	
Uninfected		1	0	1	0	100%
		2	0	1	0	
		3	0	1	0	
		4	0	2	0	
		5*	1	3	3	

Table 2: Macro scores for different *C. jejuni* strains in terms of colour, presence of bacteria in the larval gut and presence of bacteria in the larval body cavity. *G. mellonella* were incubated at different temperatures. * autolysis. A score of 3 for macro colour refers to fatality. ND = no data.

Figures



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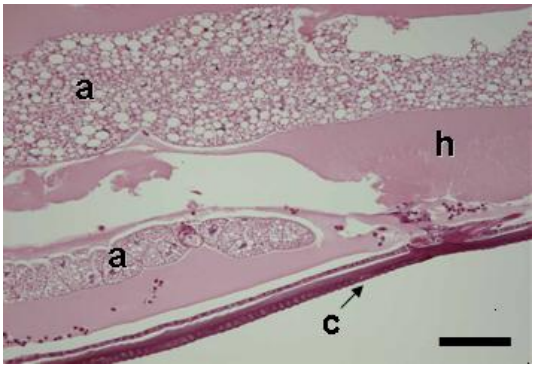

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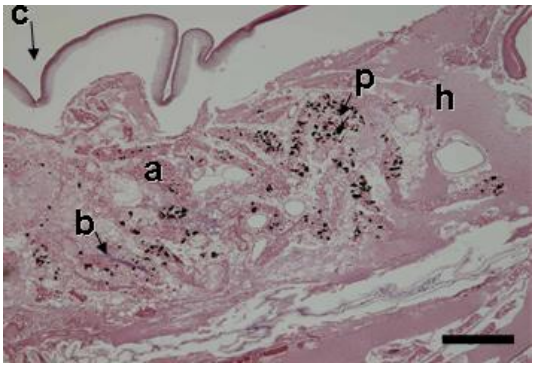

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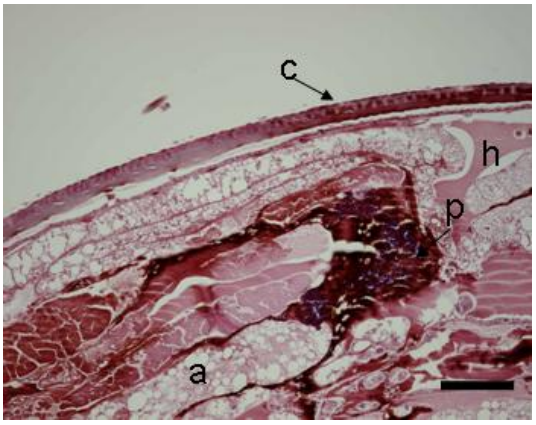


D

Figure 1: H&E stained sections of *Galleria mellonella*. (A) Non-infected larva (Bar: 100μm). (B) Control [0.1M PBS inoculation] larva. (Bar: 100μm) (C) Larva infected with *C. jejuni* 81116 and incubated at 37ºC (Bar: 250μm). (D) Larva infected with *C. jejuni* 81116 and incubated at 42ºC (Bar: 100μm). Structures annotated as follows: a – adipose bodies, b – bacterial colonies, c – cuticle, h – hemolymph, m – muscle, p – pigmented structures.

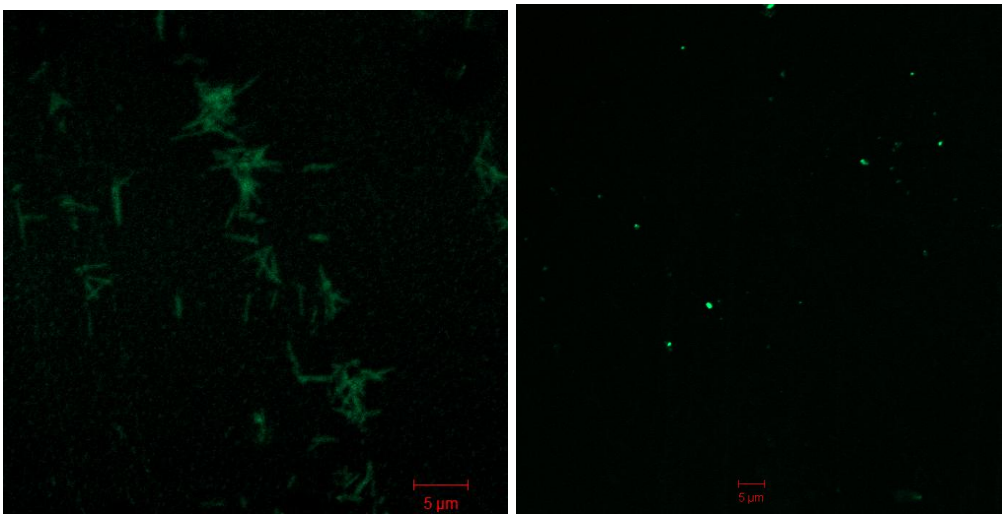
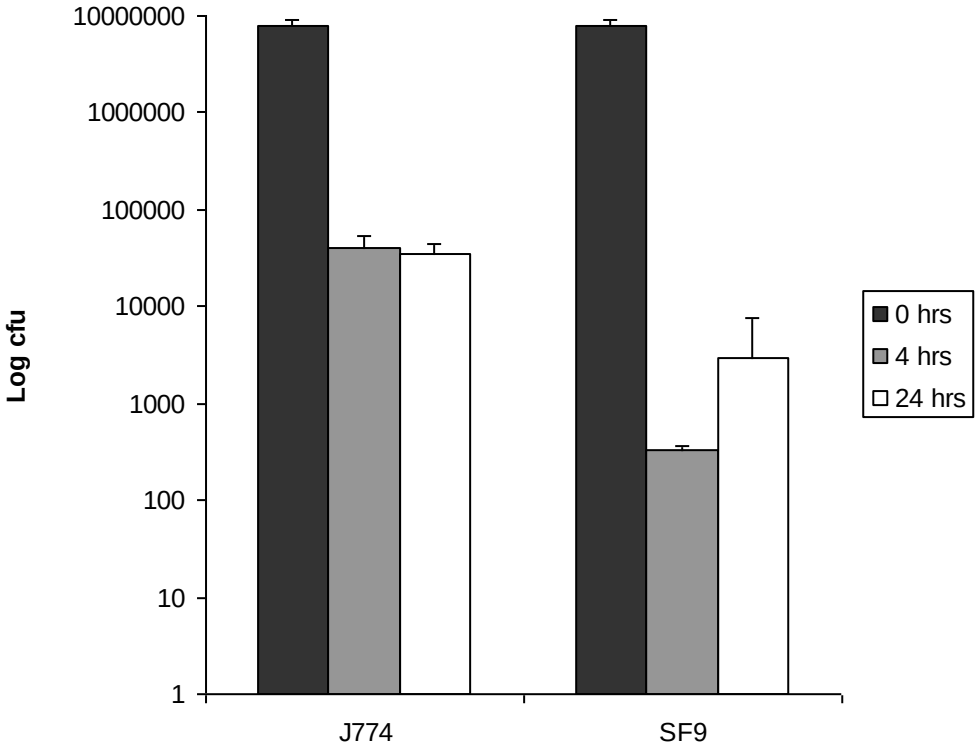


Figure 2: confocal microscope images of GFP-tagged *C. jejuni* pREM5 11168H. Left: *C. jejuni* from an overnight broth culture. Right: *C. jejuni* in the hemolymph of infected *G. mellonella* after 3 hours' incubation.

Figure 3: Macroscopic evaluation of wax moth larvae following infection with *C. jejuni*: white, score 0; orange, score 1; black, score 3

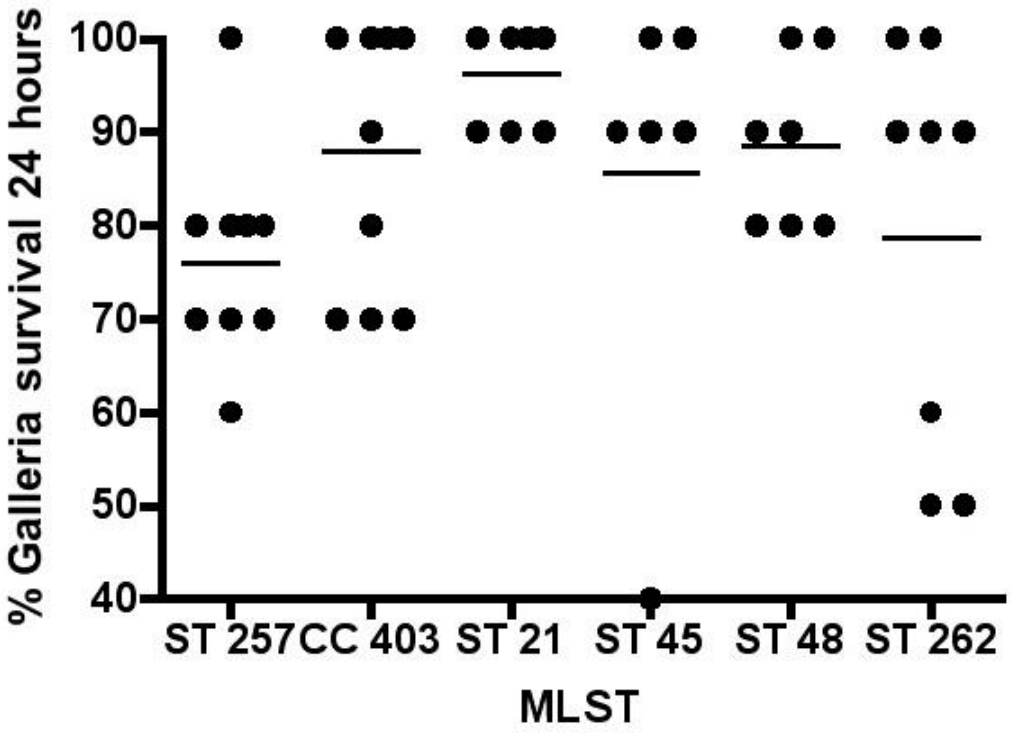


603

604 Figure 4: A chart to show the average numbers of *C. jejuni* 11168-H recovered from J774.1A murine macrophages and SF9 insect cell line at 4 hrs and 24 hrs

605 post-infection (n = 3). 0 hrs represents the initial inoculum.

606



607

Figure 5: A graph of data from MLST infections of *G. mellonella*. Each
608 spot represents the average percentage larval survival (n = 10). Horizontal lines represent the mean for each MLST type.